FEBS Letters 585 (2011) 1276–1280

journal homepage: www.FEBSLetters.org

Co-translational function of Cosmc, core 1 synthase specific molecular chaperone, revealed by a cell-free translation system

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ARTICLE INFO

Article history:
Received 8 February 2011
Revised 25 March 2011
Accepted 1 April 2011
Available online 9 April 2011

Edited by Jesus Avila

Keywords:
Core 1 synthase specific molecular chaperone
Core 1 β3-galactosyltransferase
Cell-free translation
Co-translation
O-Glycan
Molecular chaperone

ABSTRACT

The core 1 structure of the mucin type *O*-glycan is synthesized by core 1 β1,3-galactosyltransferase (C1GalT). Core 1 synthase specific molecular chaperone (Cosmc), a molecular chaperone specific for C1GalT, is essential for the expression of functional C1GalT in mammalian cells. In this study, we have established a procedure for detecting the chaperone activity of Cosmc by using a wheat germ cell-free translation system. Active C1GalT was expressed following simultaneous translation with Cosmc or translation in the presence of recombinant Cosmc protein. Moreover, we show that recombinant Cosmc must be present during the translation of C1GalT, as it is ineffective when added after translation. These results indicate that Cosmc mediates the co-translational activation of C1GalT and that it may prevent the unfavorable aggregation of C1GalT.

Structured summary of protein interactions:
C1GalT and **Cosmc** bind by enzymatic study (View Interaction 1, 2, 3, 4)

1. Introduction

O-Glycosylation is a common post-translational modification of glycoproteins and plays an important role in many biological functions [1]. Aberrant truncated *O*-glycans, such as Tn and sialyl Tn, are tumor-associated carbohydrate antigens [2]. Alterations in *O*-glycosylation have been linked to several diseases, such as IgA nephropathy and Tn syndrome [3,4]. Core 1 structure (Galβ1, 3-GalNAcα-Ser/Thr), the major constituent of *O*-glycan, is generated by the enzyme, core 1 β3-galactosyltransferase (C1GalT). C1GalT was first purified from rat liver, and its gene, *C1GalT*, has been cloned [5]. This enzyme catalyzes the transfer of galactose from UDP-galactose to a *N*-acetylgalactosamine (GalNAc) attached to Ser/Thr residues (Tn antigen) with a β1–3 linkage. The purified recombinant soluble form of C1GalT from human embryonic kidney 293T cells (HEK293T) shows core 1 synthetic activity by itself [6].

Core 1 synthase specific molecular chaperone (Cosmc) was identified as another essential protein for the expression of core 1 structure in vivo [7,8]. Some studies demonstrated that Cosmc is neither an enzyme nor a sort of subunit of C1GalT, but a molecular chaperone specific for C1GalT [6,7,9]. Cosmc supports the folding and stabilization of C1GalT in the endoplasmic reticulum (ER) [6,9]. In cells lacking Cosmc, such as the human T leukemic cell line, Jurkat, and the human colon cancer cell line, LSC, C1GalT aggregates and is subsequently degraded in the proteasome [6,8,9]. Furthermore, *Cosmc* knock-out mice [10] are embryonic lethal, owing to brain hemorrhage similar to what is observed in *C1GalT* knock-out mice [11]. Although in vivo approaches can provide functional evidence for the activity of chaperones, they do not allow careful study of the precise molecular mechanism of their action. Such studies require a highly controllable in vitro system to identify the precise role of Cosmc in the folding processes. Recently, Cosmc has been shown to promote the in vitro refolding of C1GalT [12]. The latter study only accomplished partial refolding of denatured C1Ga1T, however, and the mechanism underlying the

Abbreviations: ER, endoplasmic reticulum; C1GalT, core 1 β3-galactosyltransferase; Cosmc, core 1 synthase specific molecular chaperone; GalNAc, *N*-acetylgalactosamine; HEK293T, human embryonic kidney 293T

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doi:10.1016/j.febslet.2011.04.010

participation of Cosmc in the correct folding of C1Ga1T remains controversial.

Cell-free translation systems can be used to synthesize almost any protein, often with highly accurate folding and at a speed approaching in vivo rates. In general, however, the *Escherichia coli* cell-free system does not always successfully produce mammalian proteins that are normally secreted into the ER. A wheat germ cell-free system was recently developed for the highly efficient production of human proteins [13]. Many eukaryotic proteins can be produced in an active form by this system. [14]. Cell-free translation systems have been utilized to study chaperone function. For example, a chaperone specific for Caspase-activated DNase was shown to promote the folding of the enzyme co-translationally during its synthesis in rabbit reticulocyte lysate [15].

To shed further light on the molecular function of Cosmc, we established a cell-free translation system using wheat germ extract for the synthesis of catalytically active C1GalT, and investigated the role of Cosmc in its folding. We were able to reconstitute the expression of active C1GalT in the presence of Cosmc and we show that Cosmc is involved in the correct co-translational folding of the former.

2. Materials and methods

2.1. Construction of plasmids

The cDNAs encoding the soluble catalytic domains of human C1GalT (from Gly-32 to the C-terminus) and Cosmc (from His-37 to the C-terminus) were inserted into the in vitro transcription vectors, pEU-FLAG and pEU-His.

2.2. Preparation of mRNA

mRNAs encoding C1GalT or Cosmc were synthesized in vitro. The transcription mixtures (100 μl) contained 80 mM Hepes–KOH, pH 7.8, 16 mM magnesium acetate, 2 mM spermidine, 10 mM DTT, 3 mM of each nucleotide triphosphate (ATP, UTP, GTP and CTP), 0.8 unit/μl RNasin, 1 unit/μl SP6 RNA polymerase and 25 ng/μl plasmid. The reaction was performed at 37 °C for 2 h and the supernatant was collected by centrifugation at 13 000×*g* for 1 min, followed by ethanol precipitation.

2.3. Cell-free in vitro translation

The reaction was performed in translation buffer (1.2 mM ATP, 0.3 mM GTP, 16 mM creatine phosphate, 0.4 mM spermidine, 30 mM Hepes–KOH, pH 7.8, 0.3 mM each of 20 amino acids, 3.3 mg/ml creatine kinase, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.005% (w/v) NaN_3, and 2.6 unit/μl RNasin) with 0.4 μg/μl mRNA (0.2 μg/μl mRNA of each when two proteins were co-expressed) and wheat germ extract. The translation mixture was dialyzed against translation buffer at 26 °C for 48 h. The translated proteins in the soluble fractions were obtained by centrifugation for further analysis.

2.4. Expression of soluble N-terminal FLAG-tagged Cosmc

Recombinant human Cosmc was expressed as a secreted protein fused to FLAG-tag (293T-Cosmc). The Cosmc gene was inserted in the expression vector pFLAG-CMV3 (Sigma, St. Louis, MO) and transfected into HEK293T cells using LIPOFECTAMINE 2000 (Invit-

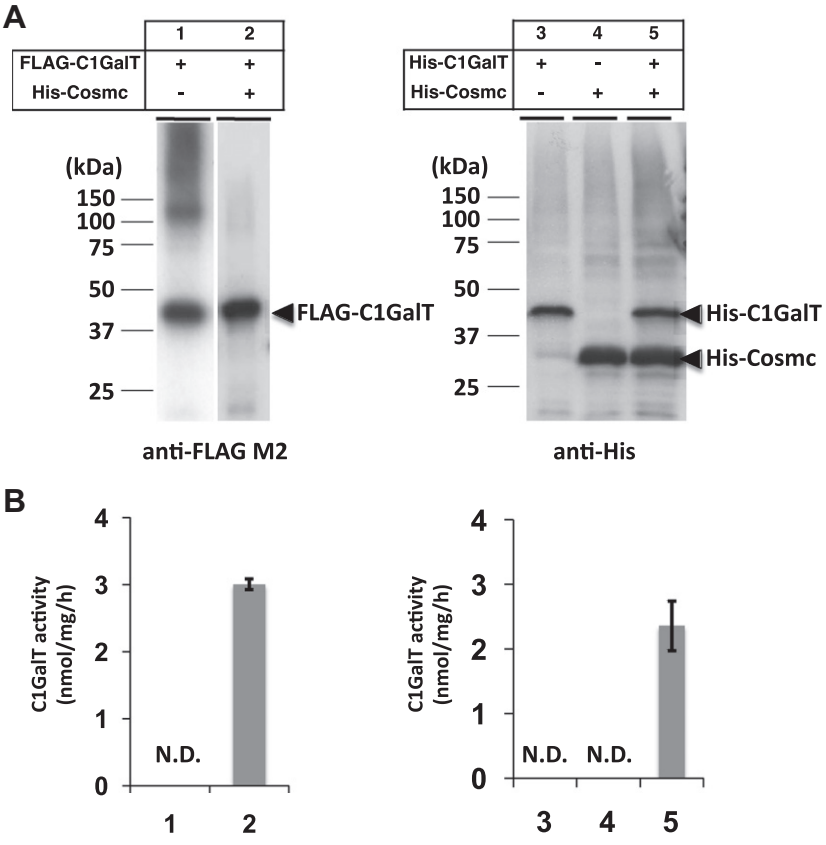

Fig. 1. Expression of catalytically active C1GalT in the wheat germ cell-free translation system. FLAG- or 6xHis-tagged human C1GalT (FLAG-C1GalT, His-C1GalT) or Cosmc (His-Cosmc) were either individually expressed (lanes 1, 3 and 4) or co-expressed (lanes 2 and 5). (A) Western blot analysis of the soluble fractions of the translation mixtures. Solid arrows indicate the position of each monomer of C1GalT or Cosmc. (B) A core 1 synthetic activity of each lane in panel A, estimated by HPLC as described in Section 2.6. Error bars represent S.D. of more than triplicate experiments.

rogen, Carlsbad, CA). 293T-Cosmc was purified as previously described [16].

2.5. SDS–PAGE and Western blot analysis

Proteins were separated by 10% SDS–PAGE and transferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was probed with peroxidase-conjugated anti-FLAG M2 antibody (Sigma) or anti-Penta-His (QIAGEN).

2.6. Enzyme reaction of C1GalT using GalNAcα-Ser-Cy5 as a substrate

The standard enzyme reaction mixture contained 50 mM Tris–HCl (pH 7.4), 5 mM $MnCl_2$, 5 mM DTT, 500 mM UDP-galactose, 5 μM Cy5 labeled GalNAcα1-Ser and the translated enzyme in a final volume of 20 μl. The reaction mixture was incubated at 37 °C for 3 h, and the reaction was terminated by boiling. The reaction products were subjected to reversed phase-HPLC using a C18 column (Waters 5C18-AR, 4.6 × 250 mm) and eluted with a gradient (15–22.5%) of acetonitrile containing 0.1% TFA at a flow rate of 1.0 ml/min at 40 °C. The Cy5-labeled product, Galβ1-3GalNAcα1-Ser-Cy5, and the initial substrate, GalNAcα1-Ser-Cy5, were detected at 670 nm (excitation, 649 nm) using a fluorescence detector, and core 1 synthetic activity was estimated by the amount of the reaction product to the total Cy5 labeled fluorescence, 100 pmol.

3. Result

3.1. Wheat germ cell-free translation of tagged C1GalT and Cosmc

For cell-free translation, we developed vectors encoding human C1GalT and Cosmc from which we synthesized the cognate mRNAs by in vitro transcription. Each construct also encoded a FLAG- or 6xHis-tag at the N-terminus of the protein, replacing the insoluble transmembrane domain that anchors the native proteins to the ER and Golgi. C1GalT and Cosmc were each translated alone from their cognate mRNAs in the wheat germ cell-free translation system and the protein products were detected by Western blot analysis using anti-FLAG and -His antibodies. Both C1GalT and Cosmc proteins were detected in the soluble fraction, with molecular mobility corresponding to those of proteins of 42 or 35 kDa, respectively (Fig. 1A, lanes 1, 3 and 4). The amount of each recombinant protein was estimated by comparing the density of the immunoreactive signals produced following anti-FLAG Western blot analysis compared to that of a quantitative control marker, FLAG-BAP (Sigma). Cell-free translation resulted in the synthesis of 10–12 μg of protein per reaction at 26 °C for 48 h, dependent upon the quantity of mRNA: 1 μg of protein from 1 μg RNA in a single reaction. However, neither C1GalT nor Cosmc exhibited core 1 synthetic activity, when the proteins were expressed alone (Fig. 1B).

Ju and Cummings demonstrated that the expression of catalytically active C1GalT in Hi-5 insect cells, which do not normally express Cosmc, required co-expression of Cosmc [7]. Therefore, we co-expressed C1GalT with Cosmc in our system. Cell-free transla-

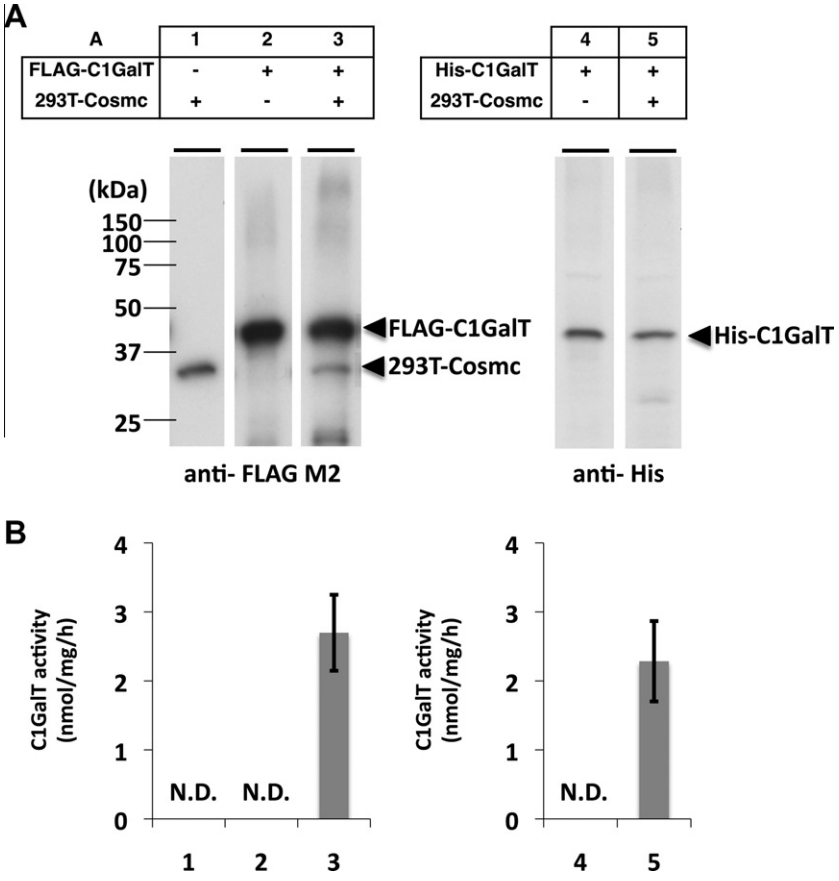

Fig. 2. Cosmc promotes co-translational folding of C1GalT. Cell-free translation of C1GalT mRNA was carried out in the absence (lanes 2 and 4) or presence (lanes 1, 3 and 5) of 293T-Cosmc. The soluble portion of the extract was subjected to Western blot analysis (A) and measurement of C1GalT enzyme activity (B). Error bars represent S.D. of triplicate experiments.

tion was performed using an equivalent amount of C1GalT and Cosmc mRNAs. As shown in Fig. 1A, lanes 2 and 5, the amount of C1GalT and Cosmc proteins that was synthesized when they were translated together was similar to that observed when they were each expressed alone. However, we were only able to detect core 1 synthetic activity when the proteins were co-translated in vitro (Fig. 1B). These results indicate that Cosmc is necessary for the generation of active C1GalT, as was reported in the in vivo studies [6,9]. In other words, the cell-free translation system reconstituted the function of Cosmc and enabled us to measure the activity of C1Ga1T as a surrogate for that of Cosmc.

3.2. Effect of Cosmc on the co-translational folding of C1Ga1T

It is still unknown how Cosmc promotes the expression of active C1GalT. One possibility is that Cosmc interacts with C1GalT during its translation. Another possibility is that Cosmc mediates activation of C1GalT following its translation. To distinguish between these possibilities, we modified the cell-free translation protocol by replacing the Cosmc mRNA with recombinant soluble FLAG-tagged Cosmc produced in HEK293T cells (293T-Cosmc).

293T-Cosmc was added to the reaction mixture containing C1GalT mRNA just before initiation of translation. The amount of C1GalT protein expressed in the presence of 293T-Cosmc was comparable to the amount expressed in the absence of 293T-Cosmc (Fig. 2A, lanes 2 and 3). This indicated that the addition of 293T-Cosmc had little effect on translation efficiency. When C1GalT was expressed in the absence of 293T-Cosmc, we were once again unable to detect core 1 synthetic activity (Fig. 2B). On the other hand, when 293T-Cosmc was added to the reaction mixture before translation, core 1 synthetic activity was observed. This result indicates that 293T-Cosmc is functional and that the simultaneous translation of the Cosmc mRNA is not necessary for its chaperone activity in the cell-free system.

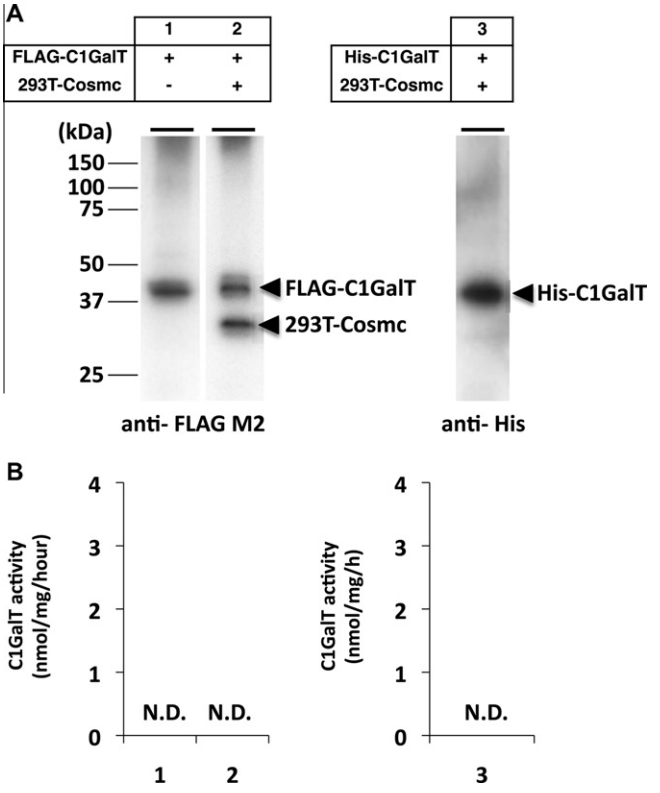

Fig. 3. Cosmc has little effect upon the post-translational folding of C1GalT. Expression of FLAG- or His-tagged C1GalT was detected by Western blot analysis (A, lane 1). 15 μg 293T-Cosmc was added into the cell-free translation mixture of C1GalT and incubated at 26 °C for 48 h. The soluble fraction of extract was subjected to Western blot analysis (A, lanes 2 and 3) and measurement of C1GalT enzyme activity (B). Triplicate experiments were performed.

3.3. Recombinant Cosmc is unable to catalyze the refolding of in vitro translated C1GalT

Another group has reported the refolding of C1GalT in vitro following the interaction of Cosmc with partly denatured C1GalT, and that this interaction partially restored the enzyme activity of the latter [12]. This indicated that Cosmc could catalyze the post-translational refolding of C1GalT.

We also investigated the refolding activity of 293T-Cosmc in the wheat germ cell-free translation system. Inactive FLAG- or 6xHis-tagged C1GalT was expressed alone in advance by cell-free translation (Fig. 3A), followed by incubation at 26 °C for 48 h in the presence of 7.9 μM 293T-Cosmc; the molar ratio of 293T-Cosmc and C1GalT was 1:1, where Comic exhibited the significant restoration of C1GalT activity in Aryal's work [12]. No degradation of C1GalT was observed following incubation in the presence or absence of 293T-Cosmc (Fig. 3B). However, we were unable to detect core 1 synthetic activity, regardless of the presence of 293T-Cosmc. Taken together, these results suggest that Cosmc mediates the co-translational folding of C1GalT before the formation of unfavorable aggregation.

4. Discussion

Although human Cosmc is reported to act as a chaperone specific for C1GalT, we investigated here more closely the precise mechanisms by which Cosmc cooperates in assisting the protein folding of C1GalT. To elucidate the molecular function of Cosmc, we established an assay system for Cosmc function based on cell-free translation using wheat germ extract. The data demonstrated that Cosmc promoted the correct co-translational folding of C1GalT but did not promote post-translational folding of the protein. However, Aryal et al. reported that Cosmc directly interacts with partly unfolded and inactive C1GalT and thereby partially restores its enzyme activity [12]. They also reported that prolonged heating reduced the amount of restoration of the enzyme activity due to aggregation of denatured C1GalT. In our study, C1GalT expressed alone by cell-free translation likely also aggregated, but not precipitated, and was not activated by the addition of recombinant 293T-Cosmc. This result is consistent with Aryal's work in that Cosmc was unable to refold C1GalT that was completely denatured and aggregated. We believe that the structural state of inactive and aggregated C1GalT proteins generated by cell-free translation is similar to that of completely denatured C1GalT generated following prolonged heating. Other groups have reported that C1GalT forms a molecular complex with Cosmc when both proteins are co-overexpressed in HEK293T or Hi5 cells [7,17]. In our experiments, we also show that C1GalT and Cosmc form a complex when C1GalT is co-expressed with Cosmc by in vitro translation (data not shown). Taken together, the results suggest that Cosmc acts co-translationally to prevent the oligomerization of C1GalT before it is able to fold correctly.

It remains unclear how Cosmc mediates the folding and activation of C1GalT. Because C1GalT forms a disulfide-bond mediated oligomeric complex in the absence of Cosmc [6], we speculate that Cosmc function may be involved in the formation of disulfide bonds with the correct pairing of cysteine residues. Site-directed mutagenesis of the cysteine residues demonstrated that mutation of two out of six cysteine residues resulted in a complete loss of enzyme activity (Supplementary). Although we were able to iden-

tify the cysteine residues of Cosmc that are key to the activation of C1GalT, further studies are needed to figure out the precise mechanism of C1GalT folding.

In the ER, there are many chaperones and folding enzymes essential for protein folding [18,19]. Mammalian secretory and membrane proteins are synthesized and translocated into the ER, where they start to fold co-translationally. The Semliki Forest virus capsid protease, for example, starts to fold immediately during translation and translocation [20]. The fusion protein, human H-Ras and mouse dihydrofolate reductase, also exhibit sequential folding of domains during synthesis [21]. Folding is also catalyzed post-translationally. The envelope glycoprotein gp160 of HIV-1 and the LDL receptor require post-translational disulfide isomerization to fold into their native structures [22,23]. An ensemble of chaperone and folding enzymes play important roles in protein folding.

In this study, we show that the expression of functional C1GalT can be reconstituted in a cell-free translation system by the addition of Cosmc, and we further show that Cosmc acts by mediating the co-translational folding of C1GalT. Cell-free translation systems are advantageous in that they can facilitate the study of novel functions of proteins. Because cell-free translation can be controlled via modification of the reaction conditions, such as by the addition of molecular chaperones or removal of inhibitory substances, they have been used to evaluate chaperone activity on the folding of synthesized proteins. Therefore, this approach may open doors to a better understanding of the molecular mechanisms of the folding of C1GalT.

Acknowledgments

This work was partly supported by the New Energy and Industrial Technology Development Organization (NEDO) and financially supported by Mitsubishi Chemical Corporation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.04.010.

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